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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-8
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	8-9
References.....	9
Appendices.....	N/A

INTRODUCTION:

Transforming Growth Factor- β s (TGF β) are polypeptides that are constitutively secreted and activated by many breast carcinomas. They contribute to the tumor's ability to invade and metastasize, to induce angiogenesis and to escape from immune destruction. These circumstances raise the question whether blocking the effects of tumor-derived TGF β on normal tissue (stroma, bloodvessels and immune cells) could be developed as a novel approach to the treatment of breast cancer. We propose to block TGF β action by developing small molecules that inhibit the type I TGF β receptor kinase, which is the key molecule that initiates and mediates TGF β signaling. We initially planned to develop a cell free ELISA-type assay for high-throughput screening for selective inhibitors of T β R-I kinase activity by using an antibody that specifically detects the phosphorylated form of its substrate, Smad2. Combinatorial libraries of small molecules would then be screened for potent and highly selective for the T β R-I kinase. These will then be tested against normal cells *in vitro* using a number of different assays for TGF β 's biological effects. Promising compounds will then be tested for their antitumor activity against highly metastatic, -angiogenic and immunogenic varieties of transplantable breast cancers in mice.

BODY:

Tasks 1 & 2. Development of high-throughput assay for specific inhibitors of T β R-I kinase and Screening of combinatorial libraries for potent and specific T β R-I kinase inhibitors

Although we had initially intended to develop a high-throughput screening assay for small molecular selective inhibitors of the T β R-I receptor kinase, and screen compound libraries ourselves, we became aware of parallel efforts by a biotech company, Scios, Inc. We were fortunate enough to be able to obtain from Scios, Inc. several small molecules that they had identified in a high-throughput *in vitro* screen as having T β R-I kinase inhibitory activity with varying degrees of potency and selectivity. This fortuitous development has allowed us to pursue Task 3 without delay.

Task 3. Pre-clinical testing of T β R-I kinase inhibitors *in vitro*

- a. Effects of TKIs on TGF β -induced Smad2 phosphorylation (Months 18-30)
- b. Effects of TKIs on reporter gene constructs (Months 18-30)
- c. Effects of TKIs on cell proliferation (Months 18-30)
- d. Effects of TKIs on extracellular matrix-associated proteins (Months 18-30)

Effects of T β R-I kinase inhibitors on TGF β signal transduction in normal cells using cell-based assays.

We reported last year that the T β R-I kinase inhibitors effectively and selectively inhibit the TGF β signal transduction pathway in whole cells *in vitro* without affecting cell viability. This conclusion was supported by the following evidence: (1) TKIs inhibited TGF β -induced phosphorylation of T β R-I's main substrates, Smad2 and Smad3. SD-093 is currently the most potent compound, with an IC₅₀ of 20 nM. (2) TKIs inhibited TGF β -mediated activation of target genes as determined in transient transfection assays using a number of different reporter gene assays. The potency of the TKIs in this regard closely paralleled their effects on Smad phosphorylation. (3) TKIs blocked TGF β -mediated growth inhibition in NMuMG normal mammary epithelial cells (MEC). (4) TKIs blocked TGF β -induced production of plasminogen activator inhibitor type 1 and collagenases, as well as ECM proteins, such as fibronectin in a dose-dependent manner. (5) TKIs blocked TGF β -induced epithelial-to-mesenchymal transdifferentiation (EMT) in normal MECs.

Effects of T β R-I kinase inhibitors on transformed mammary epithelial cells. Having established that TKIs block all of TGF β 's actions on normal mammary epithelial cells, we need to provide pre-clinical evidence that these agents might be effective as anti-cancer agents. Tumor-associated active TGF β could, in principle, provide the tumors with a selective advantage by (a) paracrine effects on supporting host cell tissues and/or (b) autocrine effects on the tumor cells themselves. Although paracrine effects can only be studied in the context of an intact animal, autocrine effects can be investigated *in vitro*. This past year, we have conducted a series of studies to determine (a) whether autocrine effects of TGF β contribute to the malignant phenotype of breast carcinoma cells

and (b) whether TKIs can be used to effectively block these actions and reverse or suppress the malignant phenotype.

Cell lines: In order to address these questions, we compared non-neoplastic mouse MECs to a series of progressively more tumorigenic and metastatic mouse mammary cancer cell lines recently generated by Dr. Susan Rittling at Rutgers University (1) (**Figure 1**). NMuMG cells are spontaneously immortalized epithelial cell line established from the normal mammary gland tissue of an adult Namru mouse using the explant method together with differential trypsinization. These cells do not form malignant lesions when injected into nude mice. Transformed MECs included: 1029 D6 (hereafter, 1029) - a cell line derived from mammary adenocarcinoma that was induced in osteopontin-deficient 129 mouse by using a Dimethylbenz(a)anthracene (DMBA)/ Medroxy Progesterone Acetate (MPA) initiation/promotion tumor induction protocol. These epithelial cells are immortal and have reduced growth factor requirements but do not grow in semi-solid media nor do they form tumors when injected into syngeneic mice. In order to achieve full transformation, Middle T antigen of polyoma virus (PMT) was expressed in the 1029 cells using a retroviral construct, giving rise to 1029 GP+E (hereafter GP+E) cells that specifically express the middle T antigen. These cells are tumorigenic *in vivo* but with a long latency, and are not metastatic. Since PMT did not confer complete tumorigenic or metastatic properties to the GP+E cells, a v-Ha-ras oncogene was transfected into these cells. Three ras expressing clones, 1029 GP+E R1, -R3 and -R5 (hereafter R1, R3, R5 respectively) were isolated. These cells are able to grow in semi-solid medium *in vitro* and are tumorigenic when injected into the mammary fat pad of syngeneic mice. In addition, R3 cell derived tumors spontaneously metastasized to lung and liver but not to bone. 8243 R3T (hereafter R3T) cells were isolated from a secondary mammary tumor and 824 R3L (hereafter R3L) cells were isolated from a lung metastasis that formed when R3 cells were injected into the mammary fat pad of syngeneic mice. Both R3T and R3L cells also formed orthotopic tumors in the mammary fat pad. Moreover, upon injection into the left cardiac ventricle of syngeneic female mice, R3T and R3L cells give rise to metastatic lesions primarily in bone. In contrast, when injected into C57Bl/6x129 F1 mice, these same cells metastasize primarily to the liver.

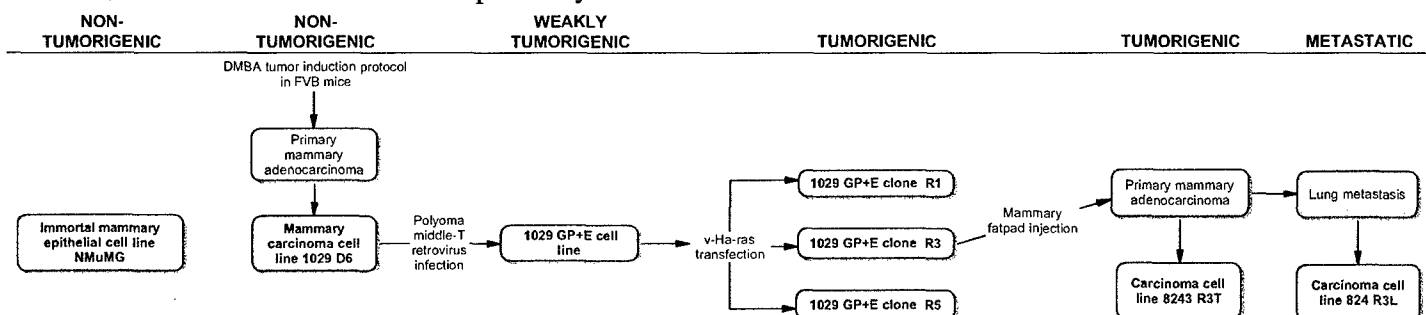


Figure 1. Overview of normal and malignant mammary epithelial cell lines used in our studies

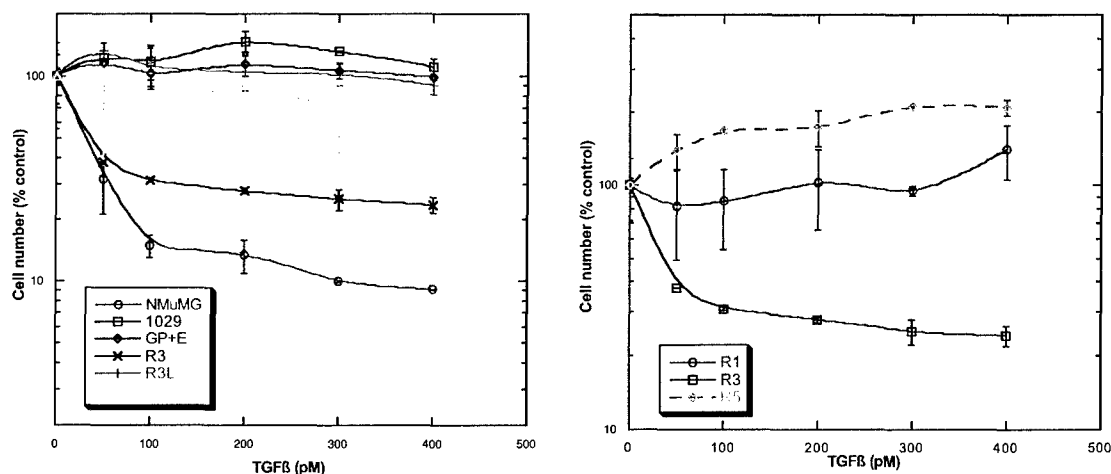


Figure 2. Cells were plated at 2×10^4 /well and incubated in the presence or absence of TGFβ (100pM) and SD-093 (1μM) for 96 h. TGFβ completely inhibited growth of NMuMG cells in a TBR-I kinase-dependent manner, while, with the exception of clone R3, all of the mammary carcinoma cells were able to proliferate in the presence of TGFβ. Values represent the mean ± SEM (n=4).

Effects of TGF β and TKIs on Anchorage-dependent Growth of Normal & Malignant Mammary Epithelial Cells: As shown in **Figure 2**, TGF β completely inhibited growth of NMuMG cells in a T β R-I kinase-dependent manner, while, with the exception of clone R3, all of the mammary carcinoma cells were able to proliferate in the presence of TGF β . Treatment of the transformed cells with the TKI SD-093 did not affect anchorage-dependent cell growth *in vitro* (not shown).

Effects of TGF β and TKIs on Epithelial-to-Mesenchymal Trans-differentiation (EMT) of Normal and Malignant Mammary Epithelial Cells: We reported last year that TGF β induces EMT in NMuMG cells, as evidenced by the acquisition of a spindle shaped morphology, loss of cell-cell contact, redistribution of E-cadherin and the formation of F-actin stress fibers (**Figure 3**). Moreover, all of these changes can be blocked by pretreating the cells with the TKI SD-093. Each of the carcinoma cell lines also displayed a typical epithelial phenotype (**Figure 3**). However, compared to NMuMG cells, the transformed cells appeared to be somewhat less cohesive and somewhat more spindly than NMuMG cells. Treatment with TGF β induced the EMT phenotype in each of the transformed lines (**Figure 3**), independently of their *in vivo* phenotype. Moreover, SD-093 treatment was able to blocked TGF β -induced EMT, and caused cells to form a monolayer of even more tightly packed polygonal cells. Assessment of F-actin organization in these cells showed that the untreated cells displayed some stress fibers even in the absence of exogenous TGF β 1 (**Figure 3**). Following TGF β 1 treatment, demarcation of F-actin bundles occurred and stress fibers appeared denser than in untreated cells. In contrast, F-actin was restored to its sub-cortical localization in SD-093-treated cells (**Figure 3**). In aggregate, these results indicate that, *in vitro*, TGF β 1 is capable of inducing EMT in both non-transformed and transformed MECs and that these changes depend on T β R1 kinase activity. Thus, in the transformed cells lines, the ability of TGF β to induce EMT has been retained, while its ability to suppress growth has been lost.

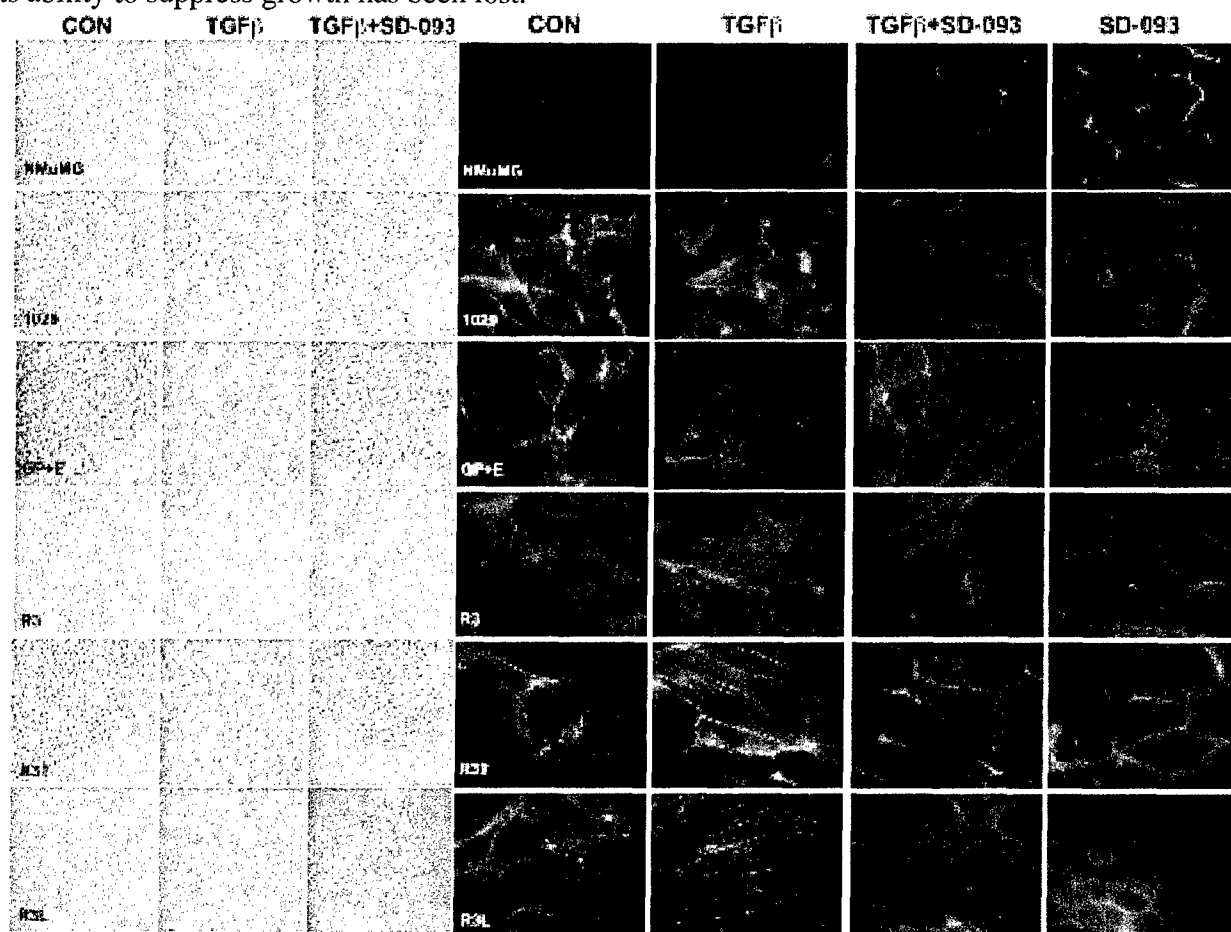


Figure 3. TGF β induced epithelial-to-mesenchymal transition (EMT) in NMuMG cells, as manifested by spindle cell morphology, reduced cell-cell cohesion, and cellular redistribution of F-actin and E-cadherin (not shown). These effects were blocked by pre-treatment with the T β R-I kinase inhibitor, SD-093. The mammary cancer cell lines displayed some degree of the EMT phenotype, even in the absence of exogenous TGF β , which was further induced by the addition of TGF β and reversed by treatment of SD-093. Cells were treated for 48h. **Left:** Phase-contrast images of live cells, 200x. **Right:** F-actin staining using Alexa Fluor 488 Phalloidin, 1000x.

Effects of TGF β and TKIs on Motility and Invasiveness of Normal & Malignant Mammary Epithelial Cells:

To assess whether the morphological alterations induced by TGF β were accompanied with functional changes, we compared its effects on cell migration and invasiveness of normal and malignant MECs (**Figure 4**). As shown in **Figure 4**, under low serum condition, TGF β did not affect NMuMG cell motility. However, cell motility was strongly stimulated (15-fold) by the addition of FBS. Interestingly, treatment with TGF β 1 completely inhibited this serum-induced motility by (**Figure 4**). Moreover, TGF β 1-induced inhibition of motility was completely reversed by pre-treating cells with SD-093. In contrast, 1029 cells were resistant to inhibition of migration by TGF β (**Figure 4**). Moreover, TGF β had a progressively stronger stimulatory effect on the migration of the tumorigenic cell lines, which was most pronounced in R3T and R3L cells (**Figure 4**). Furthermore, TGF β -stimulated migration could be blocked by SD-093, indicating that this effect was mediated by the T β RI kinase. In fact, treatment with SD-093 inhibited cell migration to a lower than basal level in the tumorigenic lines (**Figure 4**) suggesting that their motility is partially dependent on autocrine TGF β signaling even in the absence of exogenous TGF β . Finally, it should be noted that TGF β -driven motility correlated tightly with the ability of the transformed cell lines to form tumors in animals (**Figure 1**). Since TGF β enhanced motility in the transformed cells, we further sought to evaluate the effect of TGF β 1 on invasion in these cells. As shown in **Figure 4**, treatment with exogenous TGF β 1 significantly increased the ability of the R3, R3T and R3L cells to invade the GFR Matrigel® matrix, and this effect was completely inhibited by pre-treating the cells with SD-093. Thus, besides motility TGF β 1 also increased the invasive properties of tumorigenic mammary epithelial cells, and this effect is T β RI kinase-dependent. In addition, even though invasiveness of 1029 and GP+E cells was only marginally stimulated, it was strongly inhibited by SD-093. This finding indicates that even in these lines, the ability to invade Matrigel is dependent on TGF β receptor signaling.

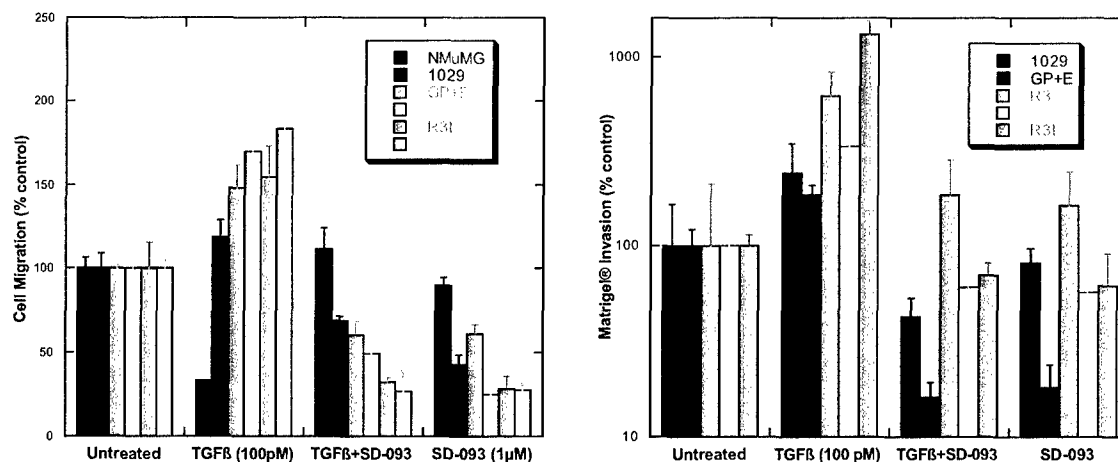


Figure 4. While serum-stimulated migration of NMuMG cells was strongly inhibited by TGF β in a T β R-I kinase-dependent manner, 1029 cells were refractory to this effect. Moreover, TGF β stimulated migration and invasiveness of the tumorigenic mammary carcinoma lines. Conversely, SD-093 inhibited migration as well as invasion of the tumorigenic lines, indicating that these processes are TGF β -dependent. Values represent the mean \pm SEM.

Effects of TGF β and TKIs on Smad2 phosphorylation in Normal & Malignant Mammary Epithelial Cells:

Finally, we examined the effects of TGF β and SD-093 on T β R-I's ability to phosphorylate its endogenous substrate, Smad2 (**Figure 5**). In non-transformed NMuMG cells, p-Smad 2 was detected only upon TGF β treatment. However, in the panel of murine carcinoma cells, particularly in R3, R3T and R3L, a low basal level of p-Smad2 was detected even in the absence of exogenous TGF β 1. Addition of TGF β 1 caused a modest increase in the levels of p-Smad2 and pre-treatment with SD-093 resulted in complete loss of phospho-Smad2 (**Figure 5**). Thus, all the mouse mammary cell lines have an intact functional TGF β receptor/Smad signaling system. Moreover, these results are consistent with my earlier observations indicating that there is a basal level of constitutive endogenous TGF β signaling present in the panel of murine carcinoma cell lines, which appears to be strongest in the most malignant lines. Most importantly, even though TGF β has acquired tumor promoting properties in the most aggressive tumor lines, the drug target (T β R-I kinase) has remained sensitive to inhibition by

the TKI SD-093. These results inspire confidence that application of these agents to tumor bearing animals *in vivo* will result in tumor regression.

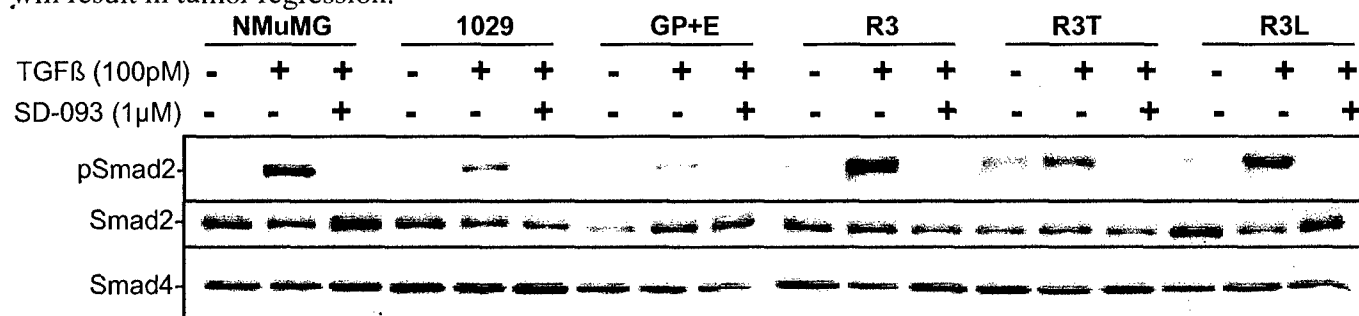


Figure 5. TGFβ induces pSmad2 and pSmad3 expression in each of the cell lines in a TBR-I-dependent manner. Moreover, R3, R3T and R3L cells expressed a basal level of pSmads even in the absence of exogenous TGFβ.

Task 4. Pre-clinical testing of TBR-I kinase inhibitors *in vivo*

- Effects of TKIs on growth of highly immunogenic transplantable mammary carcinoma lines in mice - Approximately 45 mice per compound (Months 24-36)
- Effects of TKIs on growth of highly metastatic breast cancer - Approximately 45 mice per compound (Months 24-36)
- Effects of TKIs on growth of highly angiogenic breast cancer - Approximately 45 mice per compound (Months 24-36)

KEY RESEARCH ACCOMPLISHMENTS:

- TKIs inhibit TGFβ-induced Smad2-phosphorylation in normal and malignant mammary epithelial cells
- Transformed mammary epithelial cells are refractory to TGFβ-mediated cell cycle arrest
- TKIs do not affect cell growth of transformed mammary epithelial cells
- TGFβ-inhibits motility of normal MECs but stimulates motility of tumorigenic MECs
- TKIs block TGFβ-induced motility and invasiveness of tumorigenic MECs

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Reiss, M. Transforming Growth Factor-β and cancer. In: "Cytokines in Liver Injury and Repair – Falk Symposium 125" A.M. Gressner et al. (eds). Kluwer Academic Publishers, Amsterdam. 2002. p.73-94.

Ge, R., Rajeev, V., Liu, D. and **Reiss, M.** Transforming Growth Factor-β Inhibits Cell Growth and Serum-Induced Cell Migration Through Distinct Post-receptor Signaling Mechanisms. Proc. Am. Assoc. Cancer Res. 44:In Press. 2003

CONCLUSIONS: Summarize the results to include the Importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

Late stages of breast cancer development and progression are associated with activation of TGFβ in the tumor microenvironment. This bioactive TGFβ is thought to enhance tumor progression by both autocrine effects on the tumor cells themselves and paracrine effects on stromal cells, endothelial cells, and immune cells that promote invasion, angiogenesis and escape from immune surveillance, respectively. The overall aim of this project is to develop small molecular chemical inhibitors of TGFβ signaling and test their efficacy against advanced breast cancer in animal models.

Thanks to a fortuitous collaboration with scientists at Scios, Inc., we have obtained access to small molecules that

selectively and potently target the TGF β type I receptor kinase *in vitro*. Our studies of these compounds over the past year have established that these TKIs (1) TKIs inhibit TGF β -induced Smad2-phosphorylation in whole cells without causing cell toxicity; (2) TKIs block TGF β -mediated regulation of target genes; (3) TKIs block TGF β -induced inhibition of cell growth; (4) TKIs block TGF β -mediated induction of extracellular matrix associated proteins; (5) TKIs block TGF β -induced epithelial-to-mesenchymal transition of normal mouse mammary epithelial cells.

The results of our studies from this past year indicate that TGF β undergoes a major switch during mammary carcinogenesis, from being an inhibitor of cell motility of normal MECs to becoming a stimulator of cell motility and invasiveness of tumorigenic and metastatic MECs. Moreover, TKIs inhibit motility and invasiveness of these mammary carcinoma cells. Thus, these cell lines represent a good model to test the efficacy of TKIs against mammary carcinoma cells *in vivo*.

Thus, we are poised to embark on a series of studies using animal models to test their anti-cancer effects *in vivo*, and determine whether their activity targets is primarily mediated by inhibition of metastasis or angiogenesis, or by enhancing anti-tumor immunity.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in Science, Military Medicine, etc.).

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Reiss M., TGF-beta and cancer. Microbes Infect. 1:1327-1347, 1999.

Chen, Y. and Rittling, S. R. Novel murine mammary epithelial cell lines that form osteolytic bone metastases: effect of strain background on tumor homing. Clin Exp Metastasis, 20: 111-120, 2003.